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14. ABSTRACT

The Myc oncoprotein is deregulated in the majority of breast cancers yet it has not been possible to develop a therapeutic to target Myc using traditional approaches. It has recently been shown that targeting Myc for degradation may offer a new therapeutic strategy to decrease Myc levels and kill tumor cells that are addicted to this potent oncogene. Identifying Myc protein regulatory mechanisms has been a time and labor-intensive process using classical methods to measure endogenous Myc half-life. Indeed, only one pathway has been identified to date. To facilitate this research our concept was that we could build a Myc stability probe by generating an in-frame fusion protein of Myc with a fluorescent protein (Myc-FP). After *de novo* synthesis, some FPs require hours to fold properly before they will exhibit fluorescence. Our concept was that if Myc-FP was turned over every 30 min, like Myc, then the short half-life Myc-FP would not demonstrate fluorescence, but if Myc was deregulated at the level of protein turnover, then Myc-FP would exhibit fluorescence. This switchable Myc stability probe could then be used to rapidly screen for regulators of Myc turnover. This concept has been validated and this new research tool developed, as proposed.

15. SUBJECT TERMS
Myc oncogene, anti-breast cancer therapeutics, protein turnover, high content screening, stability probe, fluorescent protein

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INTRODUCTION: Myc is a master-regulator that functions as a transcription factor to control a wide-variety of biological activities (1). In non-transformed cells Myc is highly regulated with a 20 min half-life at the level of both the mRNA and protein. By contrast, Myc expression is deregulated in over 70% of human breast cancers. Deregulated Myc expression has been shown to drive transformation in mouse models of breast cancer. Importantly, inhibiting Myc expression, using switchable MycER alleles or a dominant negative Myc, profoundly destroys existing breast cancers in the mouse. These proof-of-concept experiments provide strong evidence that targeting Myc protein in breast cancer is a potent therapeutic approach, yet targeting Myc has not been fruitful to date, leaving this potent oncogene unchecked. To fill this gap, we aim to identify the pathways that regulate Myc protein expression, at the level of protein stability, and then exploit these new insights for the development of novel anti-breast cancer therapeutics targeting oncogenic Myc (2-4).

Identifying these regulatory cascades will provide new strategies to target oncogenic Myc for destruction in breast cancer. Research tools to rapidly identify these Myc protein regulatory pathways have not been available. We aimed to explore a new and interesting concept that may offer a potential solution to this problem.

Detection of fluorescence of a given fluorescence protein (FP) is a two-step process requiring expression and then maturation (5). Most FPs used in research today have been optimized for rapid maturation in mammalian cells. However, we aim to exploit the slow (~3hr) maturation phase associated with the less commonly used FPs. Our idea is that an in-frame fusion protein, consisting of Myc-FP, will adopt the rapid turnover features of Myc. Thus, despite constitutive expression of Myc-FP protein, turnover will occur every 20 min, resulting in stable expression of the fusion protein, but no fluorescence of the Myc-FP protein. Increasing Myc half-life will enable maturation of the FP and in turn result in fluorescent Myc-FP protein. We anticipate that this Myc-FP, off/on fluorescence switch, can be used as a screening tool to identify small molecules and/or small hairpin RNAs (shRNAs) that regulate Myc stability.

Hypothesis: Our hypothesis is that Myc-FP fluorescence will be dependent upon the deregulation of Myc stability and, importantly, this research tool can be used to identify regulators of Myc turnover. By identifying the pathways and small molecules that regulate Myc, therapeutics can be developed to target deregulated Myc in breast cancer. If successful, this concept can be used to identify regulators of the many additional oncogenic short half-life proteins that play a role in breast cancer.

Objective: Evaluate whether Myc half-life is associated with Myc-FP fluorescence.

BODY: With support from the DOD, the research outlined in the original proposal has progressed in a steady and productive manner during the one year of funding. To delineate the accomplishments to date, the tasks outlined in the original Statement of Work are itemized below and a progress report for each task provided.

Statement of Work (SOW)

Task 1. ***Completed.*** After constructing and evaluating the fusion proteins composed of Myc and FP, we identified Myc-CFP (Cerulean FP) as the ideal stability probe, as outlined in the SOW. Ectopic expression of Myc-CFP in the non-transformed breast cell line MCF10A is shown in

Figure 1.

Once Myc-CFP was identified as the ideal construct, an additional construct was made to serve as a control in future stability experiments. This is composed of MycT58A-CFP. This was not originally proposed in the SOW, however we felt it was an important control that would help us interpret future experiments. Mutation of Threonine 58 to Alanine (T58A) is an established mutation of Myc that is known to increase Myc half-life from ~30min to ~60min. This was evaluated and validated in the MCF10A as shown in Figure 2.

Task 2. ***Completed.*** Protein stability and fluorescence were evaluated in the MCF10A cells expressing control or Myc-CFP fusion proteins in the absence and presence of MG132, as proposed in the SOW. First, we ectopically expressed Myc-CFP and MycT58A-CFP in MCF10A cells. We then exposed these cells to MG132 and showed that the basal level of ectopic and endogenous Myc was elevated in response to treatment (Figure 3, compare lanes 2 to 5 and 3 to 6). We then evaluated whether this increase in basal levels of Myc was due to a decrease in the rate Myc turnover following exposure to MG132. As anticipated, Myc half-life was increased in response to MG132 (Figure 4). We next evaluated fluorescence and showed that the increase in Myc-CFP stability was associated with enhanced fluorescence (Figures 5 and 6). Taken together, this provided evidence that stability was associated with fluorescence, supporting our hypothesis and working model.

We provide additional independent evidence that Myc half-life and fluorescence intensity are directly associated. This was not outlined in the original SOW, but it is standard practice to validate a positive result by an independent approach. To this end, we wanted to further support our novel concept that fluorescence of Myc_FP is dependent upon Myc turnover by extending Myc half-life in MCF10A cells by treatment with “Chiron”, a GSK-3 inhibitor. GSK-3 is an established regulator of Myc stability and thereby serves as a positive control and a second specificity control to ensure that fluorescence and Myc stability are indeed positively correlated (Figures 7 and 8). This builds on the goals and outcomes of Task 2. Our data with the Chiron inhibitor further refines the results using the proteasomal inhibitor MG132 and provides additional validation that Myc-CFP is indeed a Myc stability probe.

Task 3. ***In progress.*** We have evaluated the half-life of Myc across a panel of breast cancer cell lines (Figure 9), as proposed. We will be evaluating fluorescence in the near future. Unfortunately, this analysis was temporarily delayed due to technical difficulties associated with replacing an expensive burned-out laser in the microscope required to excite Myc-CFP. We anticipate this problem to be resolved in the coming weeks. We will then be able to compare Myc half-life across the panel of breast cancer cell lines to fluorescence intensity, as proposed.

Despite the delay in fully completing all of the initial tasks set out in the SOW, we have clearly provided experimental evidence that Myc-CFP can function as a stability probe. Thus, this concept award has lead to the development of a new research tool that we will use further in our studies to investigate uncharacterized mechanisms of Myc stability. We aim to report this observation so that other labs working on short-half life proteins, like Myc, can design similar stability probes for their research.

KEY RESEARCH ACCOMPLISHMENTS:

- Constructed new Myc-FP chimeras.

- Showed that Myc half-life and Myc-FP fluorescence are positively correlated, when ectopically expressed in the non-transformed breast MCF10A cells.

- Identified the GSK-3 inhibitor, Chiron as well as the proteasomal inhibitor MG132 as positive controls for future high-throughput screens to identify novel small molecule regulators of Myc stability.

- Measured the half-life of Myc across a panel of breast cancer cell lines. This is also new data that will be included in an upcoming manuscript once the fluorescence measurements are completed, as outlined above.

REPORTABLE OUTCOMES:

- Lindsay Lustig will graduate with a MSc and include this data as part of her thesis.

- Funding was applied for to the Canadian Cancer Society Research Institute, to use the novel Myc-CFP stability probe generated from this concept award in the development of high content screening strategies to identify regulators of Myc stability.

- Lindsay Lustig secured funding from the Canadian Breast Cancer Foundation to further support this work at the PhD level. Lindsay is a stellar graduate student who has helped to make this project a success. However, Lindsay declined the award as she has decided not to pursue a PhD at this time.

CONCLUSION: In summary, we are pleased to report that our novel concept suggesting that Myc-FP could serve as a stability probe, has been validated. The funding from this Concept Award enabled this high risk, high gain proposal to be tested in a timely manner. This stability probe fills an important gap and now provides a new research tool that will enable the regulatory mechanisms controlling Myc turnover to be identified and investigated.

To date only one pathway regulating Myc stability has been reported, yet Myc protein is deregulated in breast cancer by mechanisms that remain unclear. With this stability probe we are in a strong position to attract additional funding to conduct high content screening and rapidly identify regulatory mechanisms controlling Myc half-life in breast cancer. We can then determine the additional regulatory pathways controlling Myc and then exploit this knowledge for the development of novel therapeutics.

“So what?” Myc is deregulated in the majority of human breast cancers yet a therapeutic agent targeting Myc has not yet been generated. This is likely because Myc itself does not structurally

contain a druggable pocket that can readily be bound by traditional small molecule inhibitors. A new strategy is required. We anticipate that by understanding the regulatory pathways controlling Myc stability we will be able to target these pathways to increase Myc turnover. The tumour that is addicted to Myc deregulation will die in response to the decrease in Myc levels. This proof-of-concept has been shown with the one pathway of Myc stability that has been identified to date. By understanding the GSK-3/SCF(Fbxw7) pathway, it has become clear that targeting the deubiquitylation enzyme USP28 can deregulate and activate this pathway to enhance Myc turnover (6).

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APPENDICES: None to attach.

SUPPORTING DATA: Please see Figures.

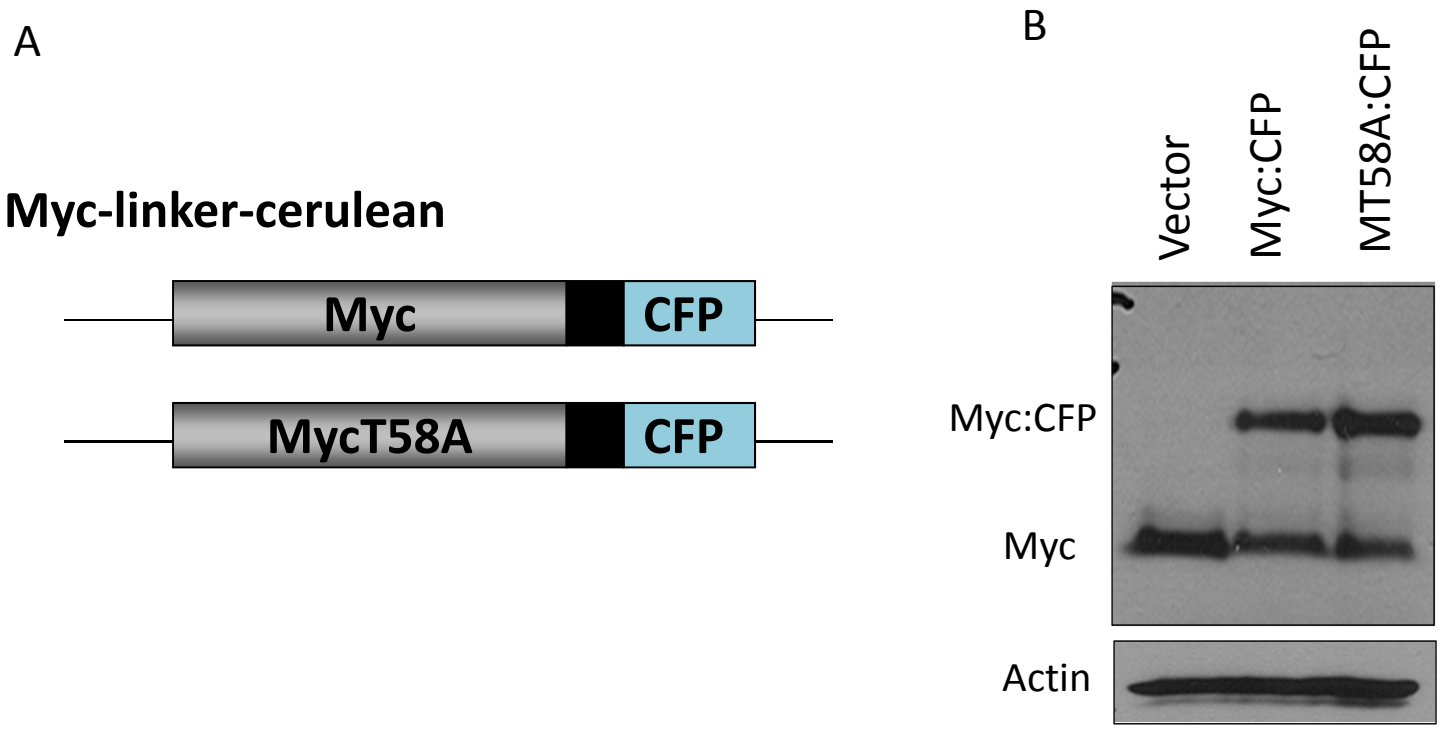

Figure 1: Myc tagged with Cerulean Fluorescent Protein (CFP) in MCF10A cells.
A) Schematic of optimal Myc-CFP fusion protein containing Myc tagged to CFP through a short linker sequence. MycT58A, a more stable Myc mutant was generated as a MycT58A-FP control. B) Western blot showing MCF10A cells retrovirally infected with empty vector control, Myc-CFP or MycT58A-CFP robustly express ectopic fusion proteins. Endogenous Myc is also evident as expected. Actin levels are shown as a loading control.

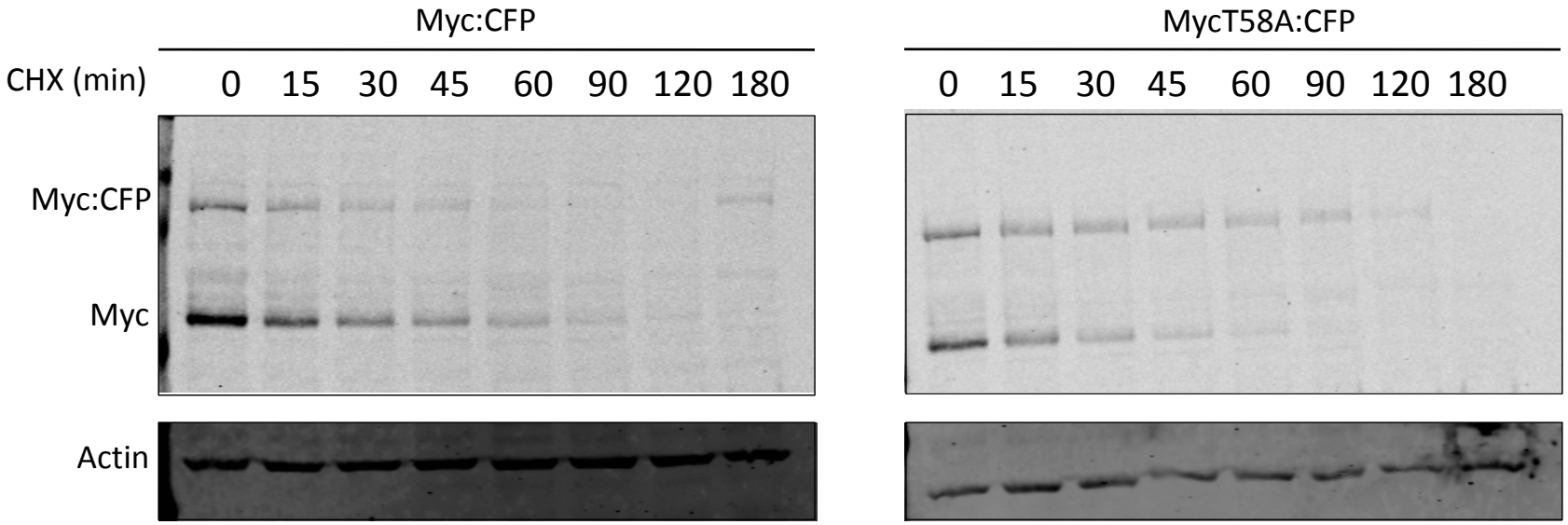

Figure 2: MycT58A mutation increases protein stability.
Immunoblot of MCF10A cells stably expressing Myc:CFP (left panel) or MycT58A:CFP (right panel). Cells were treated with cycloheximide (CHX) and harvested at the indicated times. Endogenous Myc half-life remained at 30 minutes upon ectopic expression of Myc:CFP. MycT58A:CFP ($t_{1/2}$= 60) shows a two fold increase in protein half-life compared to Myc:CFP ($t_{1/2}$= 30). Myc:CFP; ectopic Myc-FP, Myc; endogenous protein, Actin as a loading control.

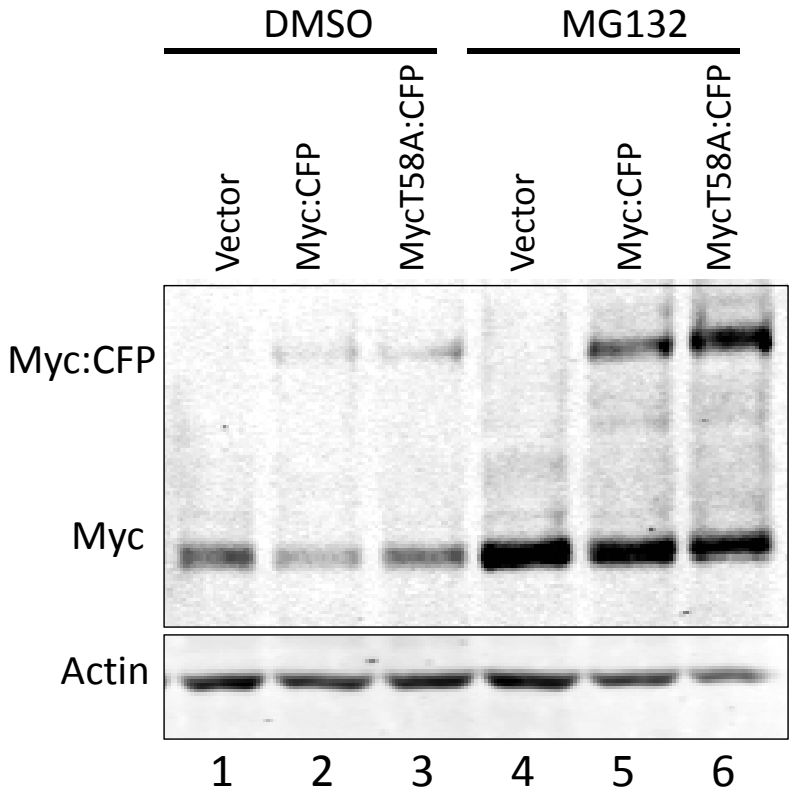

Figure 3: Proteasome inhibition increases Myc protein levels.
MCF10A cells stably expressing vector control, Myc-CFP or MycT58A-CFP were treated with 5uM MG132 or DMSO then harvested for immunoblot. Exposure to the proteasomal inhibitor MG132 resulted in a robust increase in both endogenous and ectopic Myc protein levels. Myc:CFP; ectopic Myc-FP, Myc; endogenous protein, Actin as a loading control.

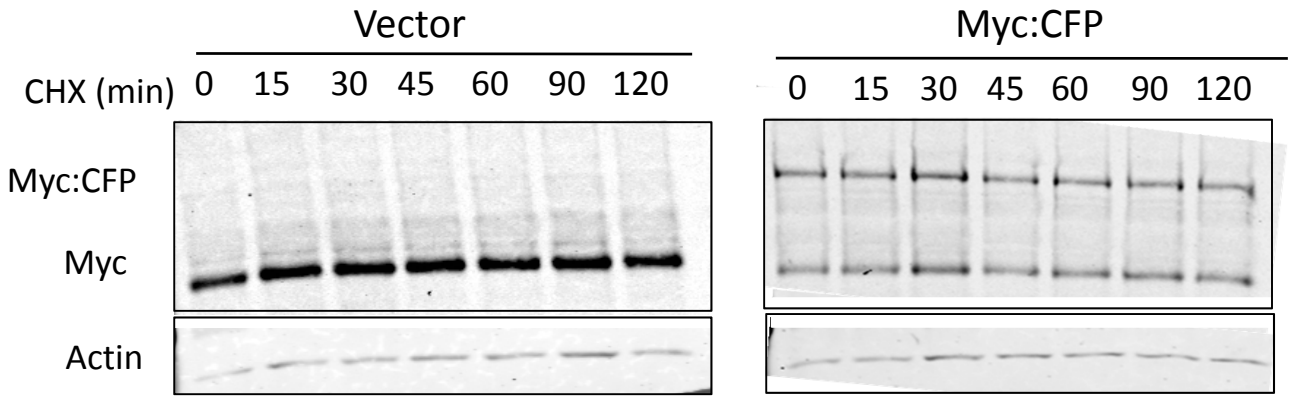

Figure 4: Proteasome inhibition increases Myc protein stability.
MCF10A cells stably expressing vector control or Myc:CFP were treated with 5uM MG132, half-life quantified following cycloheximide (CHX) exposure, then harvested for immunoblot. MG132 treatment significantly increases endogenous and ectopic Myc protein half-life. Myc:CFP; ectopic Myc-FP, Myc; endogenous protein, Actin as a loading control.

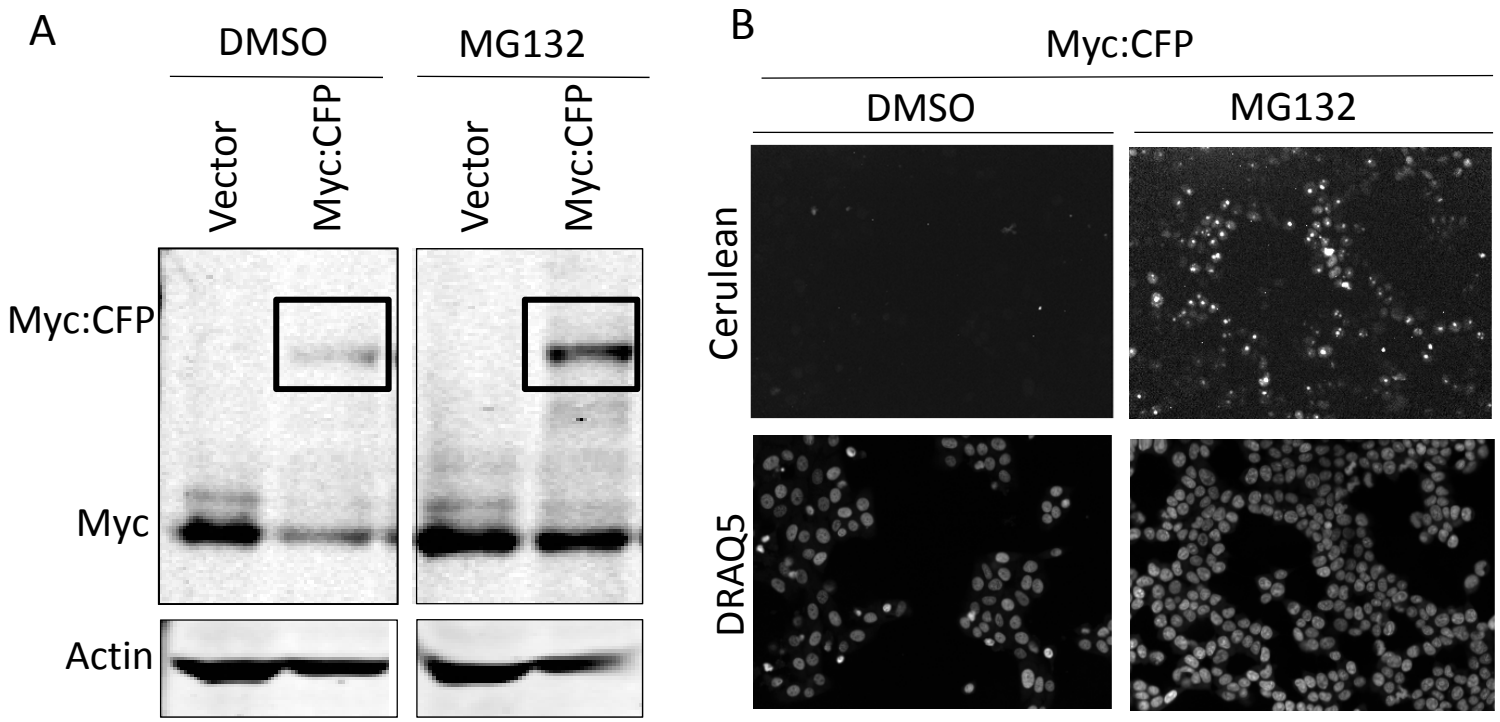

Figure 5: Proteasome inhibition increases Myc protein levels and fluorescence intensity in cells.
Vector control and Myc:CFP expressing MCF10A cells were treated with 5uM MG132 or DMSO control, (A) harvested for expression or (B) imaged using a live-cell Opera microscope. . Cerulean was excited at 430nm and emission captured through a 490 nm filter. DRAQ5 was simultaneously excited at 635 nm, and emission captured at 690 nm. Fluorescent intensity in individual cells and within the population is increased upon MG132 treatment compared to DMSO control (right panel, top). Staining cell nuclei with DRAQ5 controls for the presence of cells in the field (right panel, bottom). Left panel: Myc:CFP; ectopic Myc-FP, Myc; endogenous protein, Actin as a loading control. Right panel: Cerulean; Myc:CFP signal, DRAQ5: Nuclei.

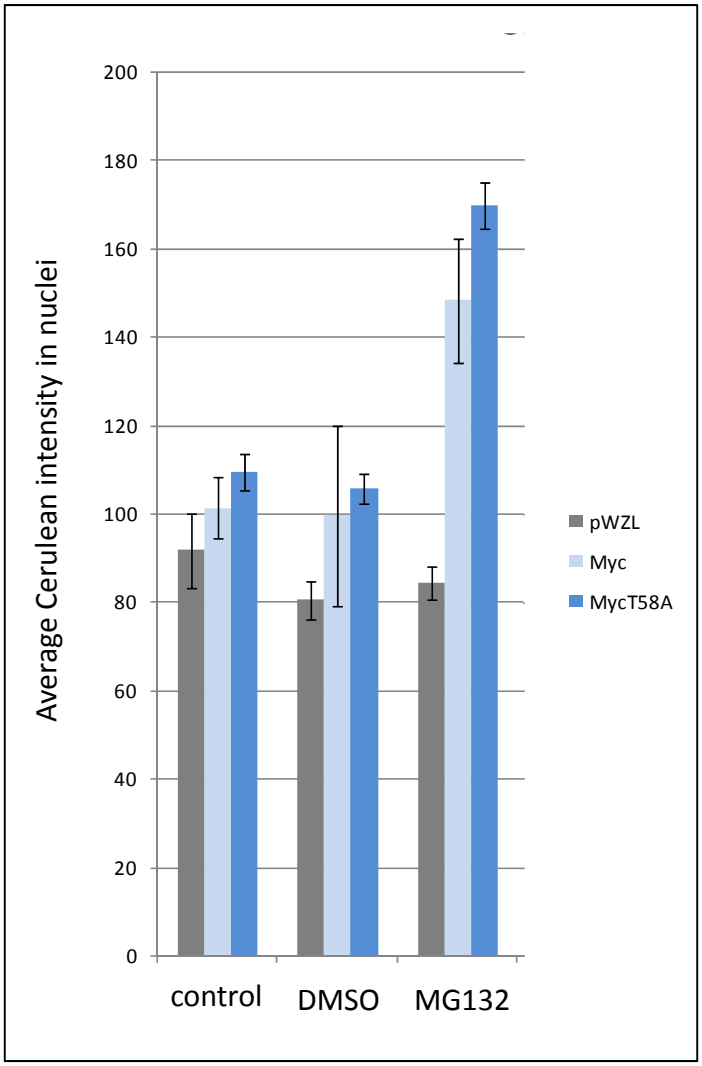

Figure 6: Increase in cerulean signal upon proteasomal inhibition.
Average cerulean intensity in nuclei was quantified for MCF10A cells stably expressing vector control (pWZL), Myc:CFP or MycT58A:CFP and either untreated (control) or treated with DMSO (vehicle control) or 5uM MG132. Intensity is scored as well mean +/- standard deviation for 8-16 wells of cells per treatment.

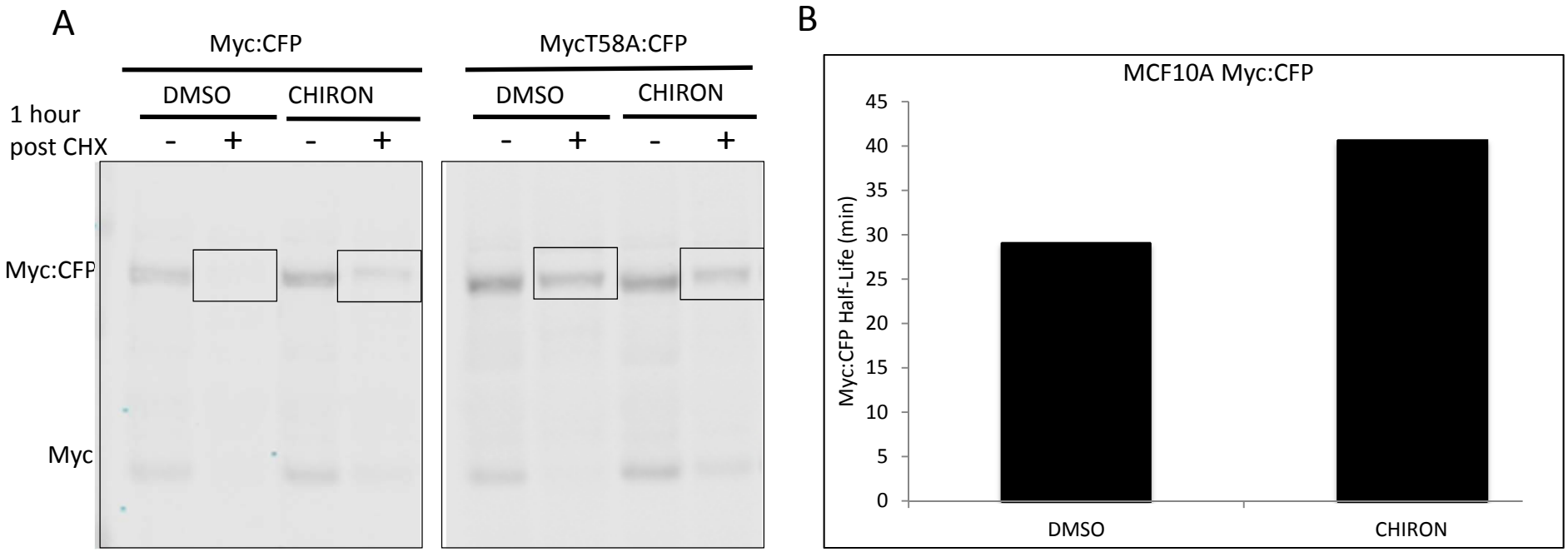

Figure 7: Inhibition of GSK-3 increases Myc:CFP stability in a T58-dependent manner. A) MCF10A cells expressing Myc:CFP or MycT58A:CFP were treated with DMSO (control) or 10uM Chiron (GSK-3 inhibitor) +/- cycloheximide (CHX) and then harvested for immunoblot analysis. Chiron acts to inhibit GSK-3; a kinase known to regulate Myc stability through residue T58. Chiron treatment increases Myc:CFP levels but not MycT58A:CFP. B) GSK-3 inhibition with Chiron increases Myc:CFP protein half-life in MCF10A cells. Quantification of traditional half-life experiments using cycloheximide is shown.

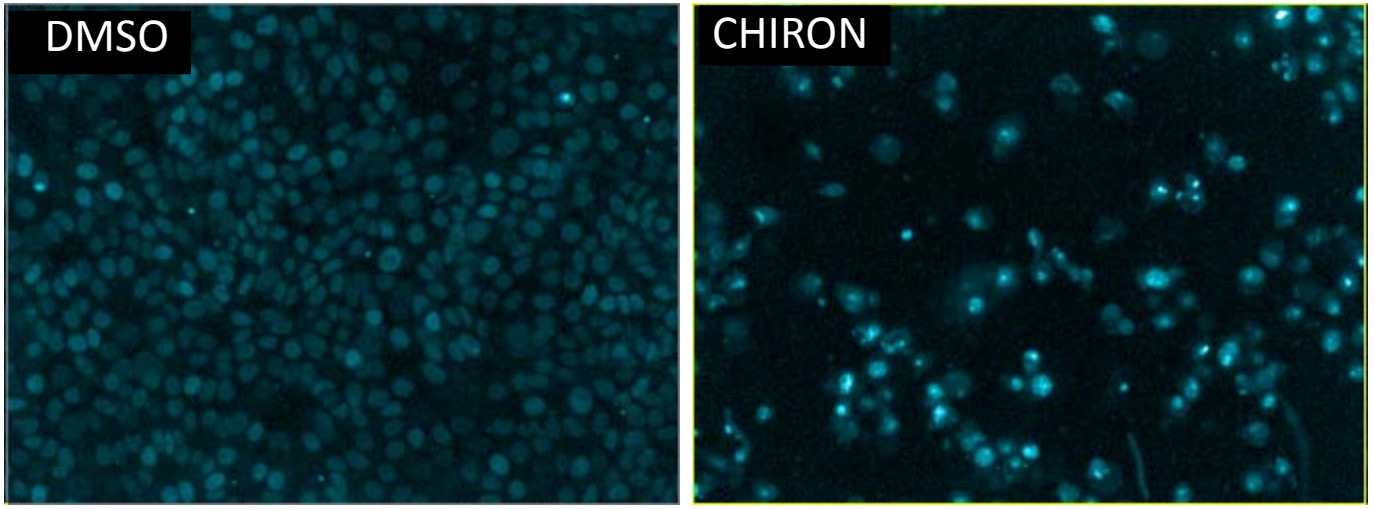

Figure 8: Treatment with chiron results in a quantifiable increase in cerulean signal. Live MCF10A cells expressing Myc:CFP were treated with DMSO (control) or Chiron (GSK-3 inhibitor) and then imaged using an Opera microscope with an environment controlled stage.

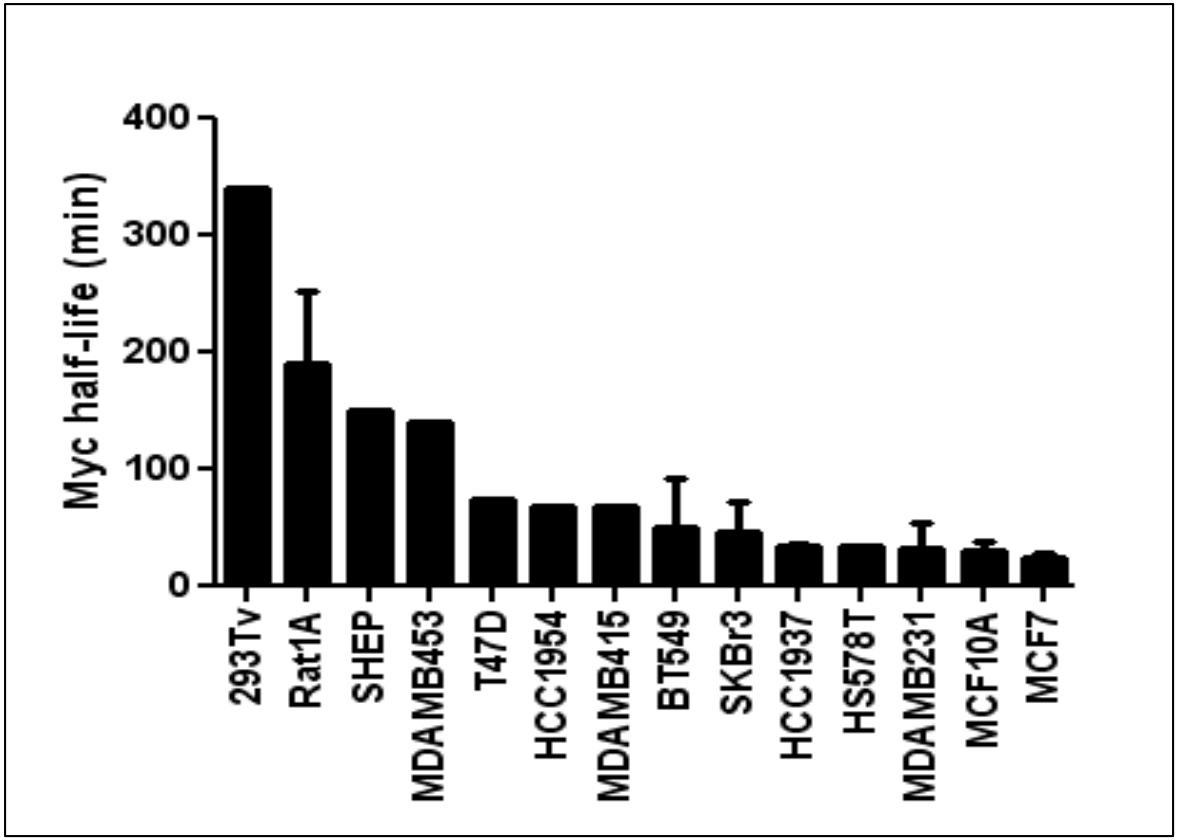

Figure 9: Myc protein stability varies between tumor-derived cell lines.
Myc protein half-life was measured using cycloheximide and immunoblot for a panel of eleven breast cancer cell lines. Stability ranges from 30 minutes, as with most non-transformed cells (e.g. MCF10A), to over 2 hrs (e.g. MDAMB-453). Three additional cell lines with long-half life Myc were also included as controls (293TV, Rat1A, SHEP).